## REMARKS

Claims 1-6 are pending in the application.

**I. The Rejections of Claims 1 and 2**

Claim 1 is rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Onishi (US Pat. 4,816,540).

Claim 2 is rejected under 35 U.S.C. 102(b) as being anticipated by Onishi (US Pat. 4,816,540).

Claim 1 is rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 1 of U.S. Patent No. 4,816,540 in view of Pack, Gene-Delivery Polymers.

Claim 2 is rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 2 of U.S. Patent No. 4,816,540.

Applicants respectfully submit that the present invention is not anticipated by or obvious over the disclosures of Onishi 4816540, alone or in view of Pack, and request that the Examiner reconsider and withdraw these rejections in view of the following remarks.

Claims 1 and 2 recite "a non-viral gene delivery vector formed of an aqueous solution of a cationic graft-copolymer..." Onishi 4816540 does not disclose or suggest such an aqueous solution.

It appears that the Examiner considers that the claimed polymer components and the polymer components of Onishi 4816540 are the same and, therefore, the products are the same. However, despite some similarities, there are clear patentable differences between the claimed non-viral gene delivery vector formed from an aqueous solution of a cationic graft-copolymer

and the cationic graft-copolymer of Onishi 4816540. Onishi 4816540 is useful as a micro carrier for cell cultivation and Immunoadsorbent. In the preparation procedure methanol was added to form an emulsion to yield a small insoluble particle. See, for example, Onishi 4816540, column 5, lines 10-11, and column 6, lines 20 and 53.

In the case of the present invention, which is useful as transfection reagent, in preparation procedure methanol was not used to form a polymer micelle. See also p. 190, ll.2-7 of the attached journal article Onishi et al, Characteristics of 2-diethylaminoethyl(DEAE)-dextran-MMA graft copolymer as a non-viral gene carrier, *Nanomedicine: Nanotechnology, Biology and Medicine*, 3,184-191 (2007).

Although the cationic graft-copolymer of Onishi 4816540 is insoluble in water, the cationic graft-copolymer of this invention is soluble in water and is thus a "non-viral gene delivery vector".

For the above reasons, it is respectfully submitted that the subject matter of claims 1 and 2 is neither taught by nor made obvious from the disclosures of Onishi 4816540, either alone or in combination with Pack, and it is requested that the rejections under 35 U.S.C. §102 and the obviousness-type double patenting rejection be reconsidered and withdrawn.

**II. The Rejections of Claims 3-6**

Claims 3 and 5 are rejected under 35 U.S.C. 103(a) as being unpatentable over Onishi (US Patent 4,816,540) in view of Pack, Gene-Delivery Polymers.

Claims 4 and 6 are rejected under 35 U.S.C. 103(a) as being unpatentable over Onishi (US Patent 4,816,540) in view of Pack, Gene-Delivery Polymers.

Claims 3-6 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 1 of Onishi (U.S. Patent No. 4,816,540) in view of Pack, Gene-Delivery Polymers.

Applicants respectfully submit that the present invention is not anticipated by or obvious over the disclosures of Onishi 4816540 in view of Pack and request that the Examiner reconsider and withdraw these rejections in view of the following remarks.

In the Office Action, at page 2, line 11, the Examiner states Onishi 4816540 teaches a latex/aqueous solution at column 1, lines 37-42. Later, at page 4, line 3, the Examiner states that Onishi 4816540 teaches a process for preparing a latex/aqueous solution.

However, at page 12, line 16, the Examiner states that Onishi 4816540 does not teach the polymer as being an aqueous solution. These descriptions are in conflict and contradictory.

On page 16, lines 22-23, of the Office Action, the Examiner states that that a latex is by definition an aqueous solution comprising a polymeric substance. It is respectfully submitted that this is not correct. Rather, a latex is by definition an aqueous emulsion comprising a polymeric substance.

Onishi 4816540 does not teach an aqueous solution. Further, Pack does not teach or disclose that using cationic polymers *in vivo* (hair, nail, tooth, skin, bone, and body) is the same as using cationic polymers as its aqueous solution.

Although the Examiner still cites Pack (Gene-delivery polymers) as teaching forming a complex between a cationic graft polymer and DNA (Section 2.2.2), again, in Pack the polymers are not graft copolymers but homopolymers, as seen by the characterization that the polymers themselves comprise linear, branched, and dendrimeric structures.

To the contrary, the cationic graft-copolymer of this invention is a new class of polycationic transfection reagents based on reacting the cationic derivative of the water-soluble linear polymer having hydroxyl groups with a polymerizable olefin monomer in the presence of a red-ox initiator. The specifically designed molecular structure of the cationic graft-copolymer having a hydrophilic-hydrophobic micro-separated-domain ensures easy entry of DNA or RNA into cells (i.e. transfection) by condensing DNA or RNA to compact structures (graft-copolymer/DNA-complex or transfection-complex) and endosome buffering. The high efficiency of the graft-copolymer makes it a valuable tool for gene delivery or gene transfer experiments. These gene delivery systems consist of an elementary step of formation of the complex between the cationic graft-copolymer so obtained and nucleic acids, such as DNA or RNA. See, for example, Applicants' specification, page 6, lines 14-23.

While a starting material/ reactant, i.e., DEAE-dextran, of the present invention is a cationic polymer similar to that cited in Pack, the cationic graft-copolymer of this invention is not a cationic polymer within the teachings of Pack.

In section 2.2, Pack also shows that various synthetic vectors, including DEAE-dextran of a starting material for this invention, have suffered from problems including toxicity, low gene transfer efficiency, and *in vivo* instability.

Pack teaches that synthetic gene delivery vectors electrostatically bind DNA and RNA (Section 2.2). Onishi 4816540 teaches that the cationic graft copolymer have a strong adsorbing power with a protein because of its cationic property and hydrophobic bond (column 1, lines 53-58). Again, Pack teaches only electrostatically bind with DNA and RNA. Therefore, even if one skilled in the art were to combine the teachings of Onishi 4816540 and Pack,

Applicants' invention would not have been obtained.

Onishi, *Nanomedicine* states "The complex form by DDMC(DEAE-Dextran-MMA-copolymer)/DNA should be a poly-ion complex (PIC) formed by a polymer micelle by the IR absorption spectrum of vOH vibration and vNH vibration at around 3100 $cm^{-1}$ to 3800 $cm^{-1}$ as shown in Figure 2. The complex by DDMC/DNA should become a more compact by shift of vNH vibration at 3450 $cm^{-1}$ in comparison with that of DDMC.

There also is a difference between the complex by DDMC/DNA and the complex by DEAE-dextran/DNA with their structure by the decrease of vNH-O vibration at around 3350 cm–1 and the shift of vNH vibration from 3450 cm–1 to 3550 cm–1 in Figures 1 and 2. This shows that the complex by DDMC/DNA should form a more compact PIC by a force from multi-intermolecule hydrogen bond and by a hydrophobic force, in comparison with the complex by DEAEdextran/DNA."

It is a misunderstanding that the grafted DEAE-dextran is able to ionicly bind anionic biopolymer as the Examiner states, because a cationic graft-copolymer of this invention is composed of a backbone polymer such as DEAE-dextran and the grafted olefin compound.

As discussed in the Remarks accompanying the Amendment filed February 27, 2008, which are hereby incorporated by reference, it is unexpected that cationic graft-copolymer so obtained by graft-polymerizing methyl methacrylate of an olefin monomer onto DEAE-dextran of a cationic derivative of a water-soluble linear polymer having hydroxyl groups can solve these problems, especially of toxicity and low gene transfer efficiency.

Pack does not provide any reason that a cationic graft-copolymer obtained by graft-polymerizing methyl methacrylate of an hydrophobic olefin monomer onto

DEAE-dextran of a cationic derivative of a water-soluble linear polymer having hydroxyl groups can solve its problems of transfection of DEAE-dextran as a cationic polymer, especially of toxicity and low gene transfer efficiency.

Pack also does not teach that the cationic graft copolymer have both its cationic property and hydrophobic bond for a strong adsorbing power with DNA or RNA.

Again, even if one skilled in the art were to combine the teachings of Onishi 4816540 and Pack, Applicants' invention would not have been obtained.

Further to the Examiner's comments on the comparative data, Figure 3 in Onishi et al, Synthesis and Characterization of 2-Diethyl-aminoethyl-Dextran-Methyl Methacrylate Graft Copolymer for Nonviral Gene Delivery Vector, *J. Applied Polymer Science*, Vol. 98,9-14 (2005) (attached) shows the transfection of a monolayer of HEK 293 cells by the DEAE–dextran–MMA graft copolymer. As shown in Figure 3, with the transfection efficiency, the DEAE–dextran–MMA graft copolymer has a maximum value in Weight increase (%). See also Onishi *J. Applied Polymer Science*, p. 12, ll. 26-33. (Weight increase (%)= (weight of MMA used/weight of DEAE–dextran hydrochloride used) $\times$ 100.)

Pack does not teach or disclose the characterization of these graft copolymer.

For the above reasons, it is respectfully submitted that the subject matter of claims 3-6 is neither taught by nor made obvious from the disclosures of Onishi 4816540 in view of Pack and it is requested that the rejections under 35 U.S.C. §103(a) and the obviousness-type double patenting rejection be reconsidered and withdrawn.

**III. Conclusion**

In view of the above, Applicants respectfully submit that their claimed invention is allowable and ask that the rejections under 35 U.S.C. §102 and 35 U.S.C. §103 and the obviousness-type double patenting rejection be reconsidered and withdrawn. Applicants respectfully submit that this case is in condition for allowance and allowance is respectfully solicited.

If any points remain at issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the local exchange number listed below.

If this paper is not timely filed, Applicant respectfully petitions for an appropriate extension of time. The fees for such an extension or any other fees that may be due with respect to this paper may be charged to Deposit Account No. 50-2866.

Respectfully submitted,

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Enclosures: Onishi et al, Characteristics of 2-diethylaminoethyl(DEAE)-dextran-MMA graft copolymer as a non-viral gene carrier, *Nanomedicine: Nanotechnology, Biology and Medicine*, 3,184-191 (2007)

Onishi et al, Synthesis and Characterization of 2-Diethyl-aminoethyl-Dextran-Methyl Methacrylate Graft Copolymer for Nonviral Gene Delivery Vector, *J. Applied Polymer Science*, Vol. 98, 9-14 (2005)

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Genetics

# Characteristics of DEAE-dextran-MMA graft copolymer as a nonviral gene carrier

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**Abstract** A stable and soapless latex of diethylaminoethyl-dextran-methyl methacrylate (DEAE-dextran-MMA) graft copolymer (DDMC) has been developed for nonviral gene delivery vectors that are possible to autoclave. DDMC relatively easily formed a polyion complex between DNA and DDMC by the hydrophobic force of graft poly(MMA) depending on its large positive entropy change ($\Delta S$). DDMC has been confirmed as having a high protection facility for DNase by DNase degradation test. Transfection activity was determined using the β-galactosidase assay, and a higher value of 16 times or more was confirmed for the DDMC samples in comparison with one of the starting DEAE-dextran hydrochloride samples. The resulting DDMC, having an amphiphilic domain so as to form a polymer micelle, should become a stable latex with a hydrophilic-hydrophobic microseparated domain. The complex of DDMC and plasmid DNA may be formed on the spherical structure of the amphiphilic microseparated domain of DDMC and have a good affinity to the cell membrane. The infrared absorption spectrum shift to a high-energy direction at around 3450 $cm^{-1}$, because of the complexes between DNA and DDMC, may cause the formation of more compact structures, not only by a coulomb force between the phosphoric acid of DNA and the DEAE group of DEAE-dextran copolymer but also by a force from the multi-intermolecule hydrogen bond in the backbone polymer DEAE-dextran and a hydrophobic force from the graft poly(MMA) in DDMC. It is thus concluded that DNA condensation may possibly have a high transfection efficiency via DDMC. The high efficiency of this graft copolymer, which is sterilized by an autoclave, may thus make it a valuable tool for safe gene delivery.


*Key words:* Nonviral gene delivery; 2-diethylaminoethyl (DEAE)-dextran-MMA graft copolymer; Transfection; DNA; Intracellular transport

Recently, in vivo gene delivery has allowed for the study of gene expression via the insertion of foreign genes or the alteration of existing genes. However, some dangerous adverse effects remain associated with the use of viral vectors. Nonviral gene delivery vectors may be a key technology in circumventing the immunogenicity inherent in viral-mediated gene transfer.

DEAE-dextran has been used as a nonviral gene delivery vector [1,2] because of its safety; it can be sterilized by autoclaving, unlike lipofection vectors. It may be preferable for gene therapy that DEAE-dextran offer only transient transfections. However, DEAE-dextran may not be superior to viral vectors and lipofection vectors with cytotoxic and transfection efficiency. For safety and a high transfection

No conflict of interest was reported by the authors of this paper.

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Table 1
Properties of DEAE-dextran-MMA graft copolymers

| Copolymer | Sample Weight-increase (%) [a] | Precipitation time by DNA (hr) |
|---|---|---|
| DDMC1 | 150 | 2.0 |
| DDMC2 | 100 | 1.0 |
| DDMC3 | 130 | 1.5 |
| DEAE-dextran | 0 | 96.0 |

[a] Sample weight increase (%) = (weight of MMA used/weight of DEAE-dextran hydrochloride used) × 100.

efficiency, many efforts have been centered on the field of nonviral gene delivery vectors, especially DEAE-dextran [3-5]. DEAE-dextran has been investigated to increase transfection efficiency [6] and found to have several good qualities for use with human macrophages [7]. DEAE-dextran, having strong adsorbing properties with DNA or RNA due to its cationic properties, has been found to change its adsorbing power for nucleic acids through pH and ion strength [8,9]. It is also well known that dextran-methyl methacrylate (MMA) graft copolymer, having a hydrophilic-hydrophobic microseparated domain, has a good affinity for the cell membrane [10]. The present article describes a novel graft copolymer having some possibilities as a nonviral gene delivery vector that is composed of a cationic derivative of dextran and a vinyl monomer. DDMC was obtained by graft-polymerizing MMA onto DEAE-dextran, in water using ceric ammonium nitrate to form a stable latex of DDMC [11-13], which is very effective as a nonviral gene delivery vector.

## Materials and methods

### *Preparation of DDMC*

Samples DDMC1, DDMC2, and DDMC3 in Table 1 were prepared as described below: 2 g of DEAE-dextran hydrochloride (nitrogen content 3%) derived from dextran having molecular weight 500,000 were dissolved in 100 mL of water, and then 4 mL, 3 mL, and 3.5 mL of MMA were added for samples DDMC1, DDMC2 and DDMC3, respectively. With stirring, the air in the reaction vessel was fully replaced with $N_2$ gas. To the solution was added 0.1 g of ceric ammonium nitrate and 15 mL of 0.1 M nitric acid, and the mixture was reacted with stirring for 1 hour at 30°C. Then, 3 mL of a 1% aqueous solution of hydroquinone were added to stop the reaction, and the resulting latex of DDMC was purified by water dialysis using a cellophane tube to remove the unreacted MMA, ceric salts, and nitric acid.

### *Reaction between DDMC and DNA*

For the reaction between DDMC and DNA, to the DNA (EX salmon sperm) solution (20 mg/mL), a solution (10 mg/mL) of the resulting latex of DDMC was added dropwise so as to obtain the complex between DDMC and DNA.

### *Characterization of DDMC*

The resulting latex of DDMC was very stable and soapless in water. The resulting DDMC precipitated by methanol became insoluble in water and acetone at 25°C. Because DEAE-dextran hydrochloride precipitated by methanol is soluble in water and poly(MMA) is soluble in acetone, it is evident that the DDMC is not a mixture but a copolymer of DEAE-dextran and poly(MMA).

The infrared (IR) absorption spectrum of DDMC as shown in Figure 1 has some characteristic absorption bands at 1730 $cm^{-1}$ and at 1000 to 1150 $cm^{-1}$, which are attributed to the carbonyl group of poly(MMA) and the pyranose ring of DEAE-dextran, respectively. Therefore, the resulting DDMC shows different solubility from DEAE-dextran and poly(MMA) and shows the above-described characteristic absorption in the IR absorption spectrum. Based on this finding, the resulting DDMC was thus considered to be a graft-polymerized compound that forms a polymer micelle.

### *Measurement of DNase degradation*

Toluidine blue (TB) and DNA form a complex by stain reaction [14]. Stain reaction between TB and DNA was carried out by adding 1 mL of 0.005% TB solution (pH 7.0) to 1 mL of DNA solution (10 mg/mL, EX salmon sperm, Wako, Osaka, Japan). For the reaction of DDMC/DNA, to 2 mL of DNA solution mixed with TB solution, the solution (10 mg/mL as DEAE-dextran) of the resulting latex of DDMC was added to obtain the complex of DDMC/DNA stained by TB.

The resulting precipitation of the complex was separated by filter (ADVANTEC 5A, Toyo Roshi, Tokyo, Japan), then added to 4 mL of distilled water, mixed with 0.01 mL (10 units) of DNase (RQ1 RNase-Free DNase, Promega, Madison, WI), and 0.1 mL of 10× reaction buffer (400 mM Tris-HCl, 100 mM $MgSO_4$, 10 mM $CaCl_2$, pH 8.0), and was incubated at 37°C for 6600 minutes. DNase degradation was determined by measuring the absorbance for TB isolated from DNA in the water with a spectrophotometer (SPECTRONIC20 MILTON ROY, Ivyland, PA). TB in water absorbs broadly in the red range, with a maximum absorption at 633 nm and a shoulder near 600 nm.The wavelength at which the absorbance was followed was chosen to give a reasonably large initial absorbance; the wavelength used for this experiment was 633 nm.

### *Transfection*

#### *Cell line and culture*

The HEK293 cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 DNA [15]. The cell line was grown in Dulbecco's Modified Eagle Medium supplemented with

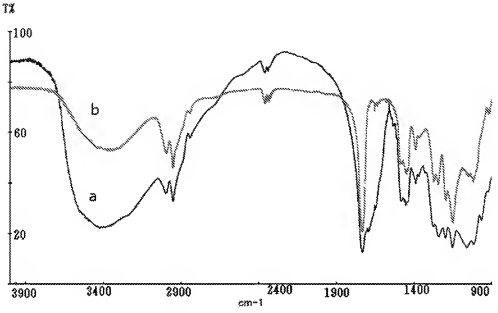


Fig 1. IR absorption spectra of DEAE-dextran-MMA graft copolymer and the complexes between DNA and DEAE-dextran-MMA graft copolymer. a, Complex of DDMC2/DNA. b, DDMC2.

10% (w/v) fetal calf serum, 0.1 mM nonessential amino acids, 5 mM L-glutamine, and antibiotics (streptomycin, 10 μg/mL; penicillin, 100 U/mL).

*Plasmid DNA*

A pCAGGS/LacZ, which expresses β-galactosidase in eukaryotic cells, was inserted under the CAG promoter of a plasmid, pCAGGS. Plasmids were amplified in *Escherichia coli* DH5α and purified using a Qiagn Mega plasmid purification kit (Qiagen, Valencia, CA).

*Transfection by DDMC/DNA*

For transfection by DDMC/DNA, HEK293 cells ($15 \times 10^4$ cells) were seeded in 35-mm culture dishes and incubated at 37°C in a humidified atmosphere of 5% $CO_2$. In a sterile tube we diluted 10 μg of DNA in 270 μL of 1 × phosphate-buffered saline (PBS). We added 14 μL of the autoclaved DDMC having a concentration of 10 mg/mL, except DDMC3 (for DDMC3, 10 mg/mL as DEAE-dextran) to the DNA solution. We then briefly mixed the solutions by vortexing. We next removed the growth medium from the cells to be transfected and washed the cells twice with 1× PBS. We then added the DDMC/DNA solution to cover the cells. We tilted the dish slowly several times to ensure complete coverage of the cells, and incubated it at 37°C for 30 minutes. We slowly tilted the dish several times during the incubation. We added 3 mL of growth medium, incubated for up to 2.5 hours or until cytotoxicity was apparent, then changed the medium and incubated it at 37°C for 48 hours. After the incubation, transfection activity was determined using the X-gal staining method and β-galactosidase assay. Following the transfection protocol, transfection of HEK293 cells by sample DDMC1, DDMC2, and DDMC3 was carried out using the plasmid DNA.

## Results

*Structure of the complex between DNA and DDMC*

The obtained complex was insoluble in water, which is a good solvent for nucleic acids. These results show that the complex between DNA and DDMC must consist of a poly-ion complex (PIC) [16] formed by a polymer micelle. In the case of sample DDMC2, the complex between DNA and DDMC2 having 100% of weight increases required 1 hour to precipitate. The complex between DNA and DDMC1 having 150% weight increases required 2 hours to precipitate, respectively. However, the complex between DNA and DEAE-dextran hydrochloride required 96 hours to precipitate at this condition, as shown in Table 1. These results show that the precipitation time (hours) decreased according to the percentage of weight increase of the DDMC samples as DDMC1 > DDMC3 > DDMC2 > DEAE-dextran. The greater the percentage weight increase of the DDMC samples, the higher the increase in hydrophobic domains in the DDMC samples by graft poly (MMA) becomes, thus making it easy to form PICs between DNA and DDMC by a hydrophobic force depending on its large positive entropy change (ΔS). Figure 1 shows the IR absorption spectra of the resulting complex between DDMC2 and DNA. The spectrum of the complex has some characteristic absorption bands at 1730 $cm^{-1}$, 1220 $cm^{-1}$, and at 1000 to 1150 $cm^{-1}$, which are attributed to the carbonyl group of poly (MMA), P-O stretching vibration of DNA, and the pyranose ring of DEAE-dextran, respectively.

Figure 2 also shows the IR spectrum of both the complex by DDMC2/DNA and the complex by DEAE-dextran/DNA in comparison with DDMC2 and DEAE-dextran, respectively. The spectrum of the complex has some characteristic absorption bands caused by hydrogen bonds by -OH and

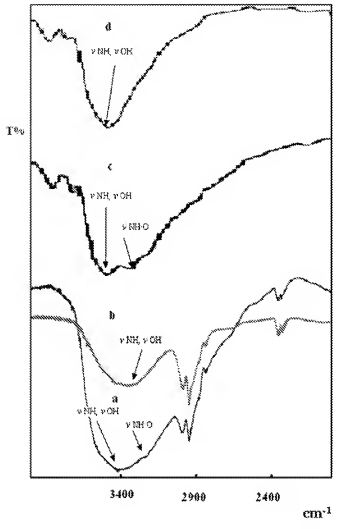


Fig 2. IR absorption spectra. a, Complex of DDMC2/DNA. b, DDMC2. c, Complex of DEAE-dextran/DNA. d, DEAE-dextran.

-NH at around 3100 $cm^{-1}$ to 3800 $cm^{-1}$ showing a structure of the complex.

The complex form by DDMC/DNA should be a PIC formed by a polymer micelle by the IR absorption spectrum of νOH vibration and νNH vibration at around 3100 $cm^{-1}$ to 3800 $cm^{-1}$ as shown in Figure 2. The complex by DDMC/DNA should become a more compact by shift of νNH vibration at 3450 $cm^{-1}$ in comparison with that of DDMC. There also was a difference between the complex by DDMC/DNA and the complex by DEAE-dextran/DNA with their structure by the decrease of νNH-O vibration at around 3350 $cm^{-1}$ and the shift of νNH vibration from 3450 $cm^{-1}$ to 3550 $cm^{-1}$ in Figures 1 and 2. This shows that the complex by DDMC/DNA should form a more compact PIC by a force from multi-intermolecule hydrogen bond and by a hydrophobic force, in comparison with the complex by DEAE-dextran/DNA.

This may be due not only to the coulomb force between the phosphoric acid of DNA and the DEAE group of DEAE-dextran copolymer but also a force from the multi-intermolecule hydrogen bond and a hydrophobic force from the hydrophobic domains of the graft poly(MMA) in DDMC. These findings lead us to conclude that DNA condensation by a coil-globule transition for DDMC may thus make it possible to obtain a high transfection efficiency [17,18].

*Transfection efficiency*

As shown in Figure 3, with the transfection efficiency, transfection activity was determined using the X-gal staining method and a higher value was confirmed for the samples of DDMC1 and DDMC2 than for the starting DEAE-dextran hydrochloride. In Figure 4, the quantities of the β-galactosidase enzyme are shown as β-gal/protein [mU·mL/mg] for the case of DDMC3 and DEAE-dextran. From these results, the transfection efficiency and the reaction rate of formation of the complex should increase by 16 times or more when using DDMC hydrochloride instead of DEAE-dextran hydrochloride.

*Cytotoxicity for the transfection*

Figure 5 shows the change of transfection efficiency when using twice the quantity of both DNA and DDMC as in the protocol, for example, 20 mg DNA. Transfection of HEK293 cells by sample DDMC1 and DDMC2, carried out using twice the quantity of both DNA and DDMC as in the protocol, has shown two times higher efficiency than the original by a transfection activity determined using the X-gal staining method. If these results are to be impossible for DEAE-dextran, cytotoxicity for the transfection should be confirmed to decrease and improve when using DDMC hydrochloride instead of DEAE-dextran hydrochloride.

*DNase degradation*

DNase I degrades both double-stranded and single-stranded DNA endonucleolytically, producing 3′-OH oligonucleotides. TB is isolated in water from DNA at the degradation, when DNA is stained by TB. Figure 6 shows the absorbance for TB isolated from the DNA of samples in the water with a spectrophotometer.

Because Beer's law plot between the absorbance and concentration of TB applies [19], Figure 6 also shows the rate of DNase degradation with a slope of both the absorbance and time. The DEAE-dextran/DNA sample shows some degradation by DNase in Figure 6, but the DDMC3/DNA sample shows a very low DNase degradation. We also found that the DNA at the colloidal stage of the interaction with DEAE-dextran was protected against DNase degradation [4,20,21]. As a result, DDMC obviously has a higher protective effect on DNase than DEAE-dextran.

## Discussion

Regarding transfection by DEAE-dextran, there were barriers to DNA delivery from degradation by DNase and dextran sucrase. To solve this problem, DDMC of a high

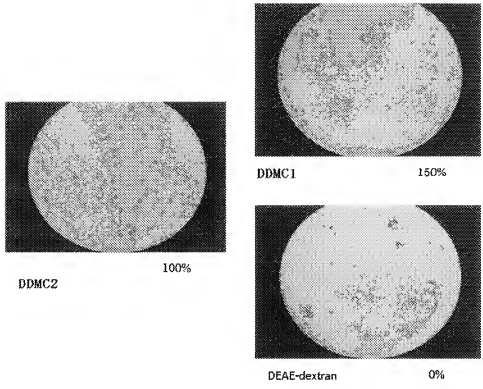


Fig 3. Transfection of a monolayer of HEK293 cells by DEAE-dextran-MMA graft copolymer. DDMC1, weight increase 150%; DDMC2, weight increase 100%; DEAE-dextran, weight increase 0%.

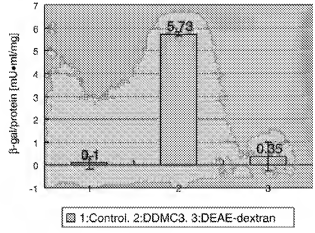


Fig 4. β-galactosidase assay. 2. DDMC3, weight increase 130%; 3. DEAE-dextran, weight increase 0%. Each condition represents the mean of four transfections per condition. Standard deviation was calculated and plotted for each condition.

protection facility for DNase was obtained by the graft polymerization of MMA onto DEAE-dextran. DDMC transfection of cells was carried out using the following steps: (1) formation of a complex between DNA and DDMC, (2) uptake, (3) endocytosis (endosome), (4) escape from endocytic vesicle, (5) DNA release in cytosol, (6) nuclear entry, (7) DNA release and transcription in nucleus.

Regarding transfection efficiency, it was considered important to examine the steps, except formation of the complex, in step (1) for DNA protection from degradation by DNase I attack, DNA penetration of the plasma membrane, and intracellular release of DNA in these steps.

However, the formation of the complex in step (1) is very important because DNA is tightly packed in native genomes and the manner of this packaging is involved in the mechanism of gene expression. Formation of the complex may induce the structural transition of DNA, which is known as DNA condensation by a coil-globule transition [17,18,22,23]. This may induce discrete ON/OFF switching in transcriptional activity [23] and may promote a protection from the degradation of entrapped DNA and associated complexes, such as the result of Figure 6. The positively charged DEAE-dextran copolymer interacts with the negatively charged phosphate backbone of DNA.

The resulting complex in step (1) is absorbed into cells by endocytosis. The specifically compact-designed molecular structure of the DDMC of a positive charge and a hydrophilic-hydrophobic microseparated domain ensures easy entry of DNA into cells for steps (2) through (7). The formation of a complex between nucleic acids (DNA or RNA) and DDMC should be accomplished at the first step by a coulomb force between the phosphoric acid of nucleic acids and its DEAE group, as explained by the IR absorption

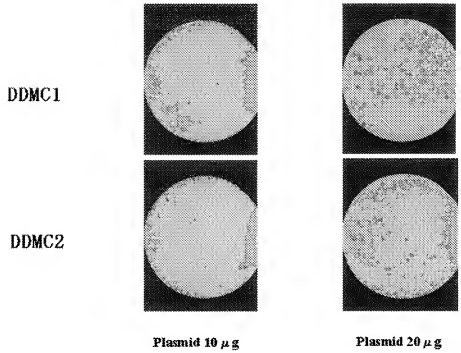


Fig 5. Effect of cytotoxicity by DEAE-dextran-MMA graft copolymer on transfection of a monolayer of HEK293 cells. DDMC1, weight increase 150%; DDMC2, weight increase 100%.

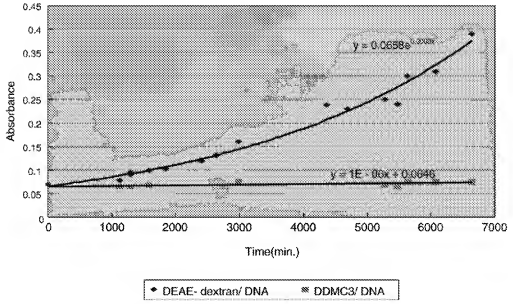


Fig 6. DNase degradation: The samples were added to 4 mL of distilled water, then 10 units of DNase (RQ1 RNase-Free DNase, Promega, Madison, WI) and 0.1 mL of 10×reaction buffer (400 mM Tris-HCl, 100 mM MgSO4, 10 mM CaCl2, pH 8), and incubated at 37°C. The wavelength used for this experiment was 633 nm for toluidine blue isolated from DNA.

spectra of the resulting complex between DDMC (sample DDMC2) and DNA in Figure 1.

The spectrum of the complex has some characteristic absorption bands at 1730 $cm^{-1}$, 1220 $cm^{-1}$, 1000 to 1150 $cm^{-1}$, and at 3450 $cm^{-1}$, which is attributed to the carbonyl group of poly(MMA), P-O stretching vibration of DNA, the pyranose ring of DEAE-dextran, and the DEAE group of DEAE-dextran, respectively. The absorption spectrum shift at about 3450 $cm^{-1}$ of the complexes may cause the formation of more compact structures by a

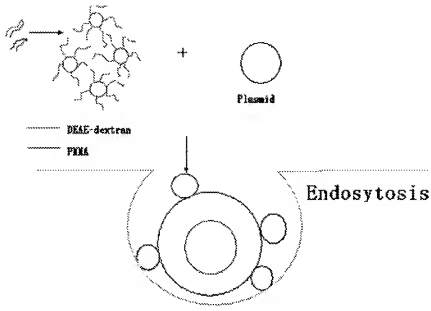


Fig 7. Schematic representation of endosytosis by a complex between DDMC and plasmid. The complex of DDMC/plasmid DNA may be formed on the spherical structure of the amphiphilic micro-separated-domain of DDMC by a polymer micelle at the first stage.

coulomb force between the phosphoric acid of DNA and the DEAE group of the DEAE-dextran copolymer to lead to DNA condensation [17,18,22,23].

However, there was also a different structure between the complex by DDMC/DNA and the complex by DEAE-dextran/DNA with the decrease of the iNH-O vibration at about 3350 $cm^{-1}$ and the shift of the iNH vibration from 3450 $cm^{-1}$ to 3550 $cm^{-1}$ in Figure 2. From the viewpoint of thermodynamics for the complex reaction between DNA and DDMC, the Gibbs free energy change at complex reaction $\Delta G[Jg^{-1}]$ can be written as follows:

$$\Delta G = \Delta H - T\Delta S \tag{1}$$

where $\Delta H[Jg^{-1}]$ is the enthalpy change and $\Delta S[Jg^{-1}K^{-1}]$ is the entropy change at the complex reaction by DNA/DDMC. The Gibbs free energy change $\Delta G[Jg^{-1}]$ at the complex reaction should be negative for its large positive entropy change ($\Delta S$) by a hydrophobic force from hydrophobic domains of poly(MMA) in DDMC, because the enthalpy change $\Delta H[Jg^{-1}]$ is very small in comparison with entropy change ($\Delta S$). This means that the complex reaction between DNA and DDMC may easily proceed and the molecule of the complex in a crystal structure may be more tightly packed in comparison to DEAE-dextran/DNA, as shown by its precipitation time in Table 1.

This may support the concept that the complex by DDMC/DNA is compactly formed not only by a coulomb force between the phosphoric acid of DNA and the DEAE group of DEAE-dextran copolymer but also by a force from the multi-intermolecule hydrogen bond and a hydrophobic force from the hydrophobic domains of graft poly(MMA) in DDMC, and that it has a high protection facility for DNase, as shown in Figure 6.

As shown in Figure 7, the resulting DDMC of hairy nanoparticles, which has an amphiphilic domain so as to form a polymer micelle, should become a stable latex of core shell particles with a hydrophilic-hydrophobic microsepa-rated domain [24]. The complex of DDMC and plasmid DNA may be formed initially on the spherical structure of the amphiphilic microseparated-domain of DDMC and have a good affinity to the cell membrane. When (step 5) DNA is released in cytosol, the spherical structure may be protected from degradation by DNase and dextran sucrase,thereby making it easier for (step 6) nuclear entry to occur.

## Conclusions

Based on the above findings, DDMC is therefore considered to have a reasonably high protective effect on DNase, a low cytotoxicity, and a high transfection efficiency.

The high efficiency of this autoclaved graft copolymer may thus make it a valuable tool for gene delivery in vivo.

Figure 7. Schematic representation of endocytosis by a complex between DDMC and plasmid. The complex of DDMC/plasmid DNA may be formed initially on the spherical structure of the amphiphilic microseparated domain of DDMC by a polymer micelle.

## Acknowledgment

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## References


[1] Murata J, Ohya Y, Ouchi T. Possibility of application of quaternary chitosan having pendant galactose residues as gene delivery tool. Carbohydr Polym 1966;29:69-74.

[2] Sato T. Carbohydrate polymer for gene delivery. Kobunshi 2002;51: 837-40.

[3] McCutchan JH, Pagano JS. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J Natl Cancer Inst 1968;41:351-8.

[4] Warden D, Thorne HV. Influence of diethylaminoethyl-dextran on uptake and degradation of polyoma virus deoxyribonucleic acid by mouse embryo cells. J Virol 1969;4:380-7.

[5] Constantin T, Vendrely C. Effect of DEAE-dextran on the incorporation of tritiated DNA by cultured rat cells. CR Soc Biol 1969;163: 300-5.

[6] Graha FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 1973;52: 456-67.

[7] Mack KD, Wei R, Elbagarri A, Abbey N, McGrath SD. A novel method for DEAE-dextran mediated transfection of adherent primary cultured human macrophages. Immunol Methods 1998;211: 79-86.

[8] Onishi Y, Kikuchi Y. Study of the complex between DNA and DEAE-dextran. Kobunshi Ronbunshu 2003;60:359-64.

[9] Onishi Y, Kikuchi Y. Study of the complex between RNA and DEAE-dextran. Kobunshi Ronbunshu 2004;61:139-43.

[10] Onishi Y, Maruno S, Kamiya S, Hokkoku S, Hasegawa M. Preparation and characteristics of dextran-methyl methacrylate graft copolymer. Polymer 1978;19:1325-8.

[11] Onishi Y. Cationic graft-copolymer. U.S. Patent 4816540 (1987).

[12] Onishi Y, Eshita Y, Murashita A, Mizuno M, Yoshida J. Synthesis and characterization of 2-diethylaminoethyl(DEAE)-dextran-MMA graft copolymer for non-viral gene delivery vector. J Appl Polym Sci 2005; 98:9-14.

[13] Higashihara J, Onishi Y, Mizuno M, Yoshida J, Tamori N, Dieng H, et al. Transfection of foreign genes into culture cells using novel DEAE-dextran copolymer as a non-viral gene carrier (Abstract 15). The 55th annual meeting of southern region, the Japan Society of Medical Entomology and Zoology, Miyazaki Prefecture, Japan, 23 October 2005.

[14] Schreier JB. Modification of deoxyribonuclease test medium for rapid identification of Serratia marcescens. Am J Clin Pathol 1969;51:711-6.

[15] Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59-74.

[16] Michaels AS. Polyelectrolyte complexes. Ind Eng Chem 1965;57: 32-40.

[17] Bloomfield VA. DNA condensation by multivalent cations. Biopolymers 1997;44:269-82.

[18] Yoshikawa Y, Emi N, Kanbe T, Yoshikawa K, Saito H. Folding and aggregation of DNA chains induced by complexation with lipospermine: formation of a nucleosome-like structure and network assembly. FEBS Lett 1996;396:71-6.

[19] Ogren PJ, Henry I, Fleicher SES, Kelly I. Chemical applications of a programmable image acquisition system. J Chem Educ 2003;80: 699-703.

[20] Maes R, Sedwick W, Vaheri A. Interaction between DEAE-dextran and nucleic acids. Biochim Biophys Acta 1967;134:269-76.

[21] Pagano JS, McCutchan JH, Vaheri A. Factors influencing the enhancement of the infectivity of poliovirus ribonucleic acid by diethylaminoethyl-dextran. J Virol 1967;1:891-7.

[22] Chaszczewska-Markowska M, Ugorski M, Langner M. Plasmid condensation induced by cationic compounds: hydrophilic polylysine and amphiphilic cationic lipid. Cell Mol Biol Lett 2004;9:3-13.

[23] Yoshikawa Y, Tsumoto K, Yoshikawa K. Switching of higher-order structure of DNA and gene expression. Seibutsu Butsuri 2002;42: 179-84.

[24] Price C, Woods D. A method for studying micellar aggregates in block and graft copolymers. Euro Polymer Sci 1973;9:827-30.

# Synthesis and Characterization of 2-Diethyl-aminoethyl–Dextran–Methyl Methacrylate Graft Copolymer for Nonviral Gene Delivery Vector

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**ABSTRACT:** A stable and soapless latex of 2-diethyl-aminoethyl (DEAE)–dextran–methyl methacrylate (MMA) graft copolymer (DDMC) was developed for nonviral gene delivery vectors (complex between polycation and nucleic acid). DDMC was newly prepared using MMA and DEAE–dextran. Following a transfection protocol, transfection of HEK 293 cells by DDC1, DDC2, and DDC3 samples was carried out using plasmid DNA. With the transfection efficiency determined using the X-Gal staining method, a higher value of 5 times or more was confirmed for DDMC samples DDC1 and DDC2 (but not for DDC3) than for the starting DEAE–dextran hydrochloride. The absorption spectrum shift at around 3400 $cm^{-1}$ of the complexes between DDMC and DNA may support the formation of more compact structures by a Coulomb force between the phosphoric acid of DNA and the DEAE group of DEAE–dextran, concluding in DNA condensation. The specifically designed molecular structure of DDMC to ensure easy entry of DNA into cells needs not only a positive charge and a hydrophilic–hydrophobic microseparated domain but also more compact structures for transfection steps. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 98: 9–14, 2005

**Key words:** transfection; DNA; 2-diethyl-aminoethyl–dextran; methyl methacrylate; positive charge; polyion complex; microseparated domain

## INTRODUCTION

Recently, *in vivo* gene delivery has allowed the study of gene expression and function in animal models via insertion of foreign genes or alteration of existing genes and/or their expression patterns. The transfection mechanism between transferred DNA or RNA and a cell has been studied and clinical tests for transfection have become easy to carry out using a viral vector. However, some dangerous adverse effects remain associated with the use of viral vectors.

Nonviral gene delivery vectors may be a key technology in circumventing the immunogenicity inherent in viral-mediated gene transfer.

Water-soluble cationic polysaccharides are also of interest for a nonviral gene delivery vector to increase safety by minimizing the incidence of serious diseases resulting from the immunogenicity inherent in viral vectors. 2-Diethyl-aminoethyl (DEAE)–dextran has been used for a nonviral gene delivery vector.[1–3] However, these cationic polysaccharides, such as DEAE–dextran, are not superior to viral vectors with transfection efficiency.

Many efforts have been made for safety and high transfection efficiency in the field of nonviral gene delivery vectors.[4–6] DEAE–dextran has been investigated, and its transfection conditions increase transfection efficiency and several good conditions for a human macrophage have been found.[7]

DEAE–dextran has strong adsorbing properties with nucleic acids, such as DNA and RNA, because of its cationic properties and is able to adsorb specific nucleic acids by changing the pH and ion strength.[8,9]

The interaction between DNA and basic proteins such as histones, known by the appearance of a partially unfolded part on chromatin, plays a key role in the regulation of the gene transfer system.[10] The structural transition of DNA, which is called a coil–globule transition, induces discrete on/off switching on transcriptional activity.[11] This collapse transition in single giant DNA chains has been reviewed as DNA condensation.[12] The *in vitro* collapse of DNA may be induced by various cationic compound vectors such as cationic lipids,[13–15] peptides,[16] or cationic polymers.[17,18] In the case of cationic lipid vectors, the complex of dioctadecylamidoglycylspermine (DOGS)/DNA, which has a nucleosome-like structure in which DNA wraps around a micellar aggregate of DOGS and has an

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association with each other to form a network structure, is very effective for gene transfection.[11]

The complex by cationic polymers/DNA in cytoplasm can be protected from restriction enzymes for the collapse of DNA.[12] In the case of cationic dextran, the complex of DEAE–dextran/DNA in the cytoplasm can be protected from DNase.[5] However, there are problems for the complex to be degraded *in vivo* by dextranase.

DEAE–dextran must have a high facility for endocytosis. However, its transfection efficiency is not so high but the reasons why may not be enough to protect it from degradation of the complex between DEAE–dextran and DNA, especially at the escape from the endocytic vesicle.[3]

Graft polymerization of methyl methacrylate (MMA) onto DEAE–dextran can be very effective for the improvement of the defect of DEAE–dextran with its protective facility from the degradation of the complex in the cytoplasm because of its graft chains of MMA.[19]

These graft copolymers have an amphiphilic domain to form a polymer micelle and should become a stable latex with a hydrophilic–hydrophobic microseparated domain to form a spherical structure.[20] The complex of DEAE–dextran–MMA graft copolymer (DDMC)/DNA to be formed on the spherical structure of the amphiphilic microseparated domain of DDMC should be stable for intracellular surroundings and have a good affinity to the cell membrane because of its hydrophilic–hydrophobic microseparated domain.[21]

The present article is related to a novel graft copolymer having some possibilities as a nonviral gene delivery vector that is composed of a cationic derivative of a water-soluble linear polymer and a vinyl ester monomer.

The DDMC graft copolymer was obtained by graft polymerizing a vinyl ester monomer (MMA) onto a cationic derivative of a water-soluble linear polysaccharide (DEAE–dextran) in water using ceric ammonium nitrate to obtain a stable latex of DDMC,[22] which is very effective as a nonviral gene delivery vector.

It is expected that nonviral vectors, such as the DDMC in this article, will increase safety by minimizing the incidence of serious diseases resulting from the immunogenicity inherent in viral vectors.

## EXPERIMENTAL

### Polymerization procedure of DDMC

Samples DDC1, DDC2, and DDC3 in Table I were prepared by the following procedure: 2 g of DEAE–dextran hydrochloride (3% nitrogen) derived from dextran (weight-average molecular weight = 500,000) was dissolved in 100 mL of water; then, 3, 4, and 6 mL of MMA was added for DDC1, DDC2, and DDC3 samples, respectively. While stirring, the air in the reaction vessel was fully replaced with nitrogen gas. To the solution was added 0.1 g of ceric ammonium nitrate and 15 mL of 0.1*N* nitric acid, and the mixture was reacted with stirring for 1 h at 30°C. Then, 3 mL of a 1% aqueous solution of hydroquinone was added to stop the reaction, and the resulting latex of DDMC was purified by water dialysis using a cellophane tube in order to remove the unreacted MMA, ceric salts, and nitric acid. The resulting latex of DDMC was stable and soapless.

**TABLE I**
**Properties of DEAE–Dextran–MMA Graft Copolymers**

| Sample | Weight increase (%) | Precipitation time by DNA (h) |
|---|---|---|
| DDC1 | 150 | 2.0 |
| DDC2 | 200 | 1.0 |
| DDC3 | 300 | 0.5 |
| DEAE–dextran | 0 | 96.0 |

Weight increase (%) = (weight of MMA used/weight of DEAE–dextran hydrochloride used) × 100.

### Synthesis of complex of DDMC/DNA

Two milliliters of a 10 mg/mL solution of the resulting latex of DDMC was added dropwise to 1 mL of a 20 mg/mL DNA (EX salmon sperm) solution to obtain a complex of DDMC/DNA.

### Measurement of IR absorption spectra

IR measurements on DDMC samples and DDMC/DNA complexes were carried out by the KBr powder method using Jasco FT/IR-300.

### Transfection protocol

The protocol of DDMC for transfection of monolayer cells is a modification of the protocol by Al-Mosfih and Dubes.[23]

### Cell line and cell culture

The 293 cell line is a permanent line of primary human embryonal kidney (HEK) transformed by sheared human adenovirus type 5 DNA. The cell line was grown at 37°C in the presence of 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.1 m*M* nonessential amino acids, 5 m*M* L-glutamine, and antibiotics (100 μg/mL streptomycin, 100 U/mL penicillin).

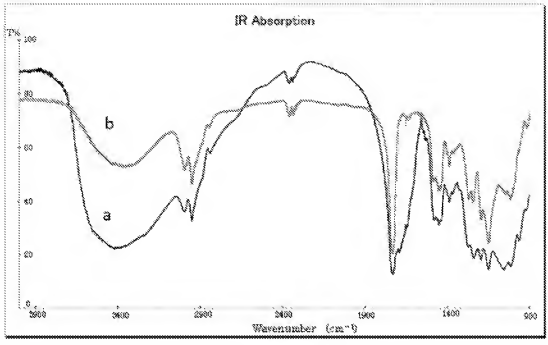


**Figure 1** IR absorption spectra of DEAE–dextran–MMA graft copolymer and the complexes between DNA and DEAE–dextran–MMA graft copolymer: DDC2/DNA complex (spectrum a) and DDC2 (spectrum b).

### Plasmid DNA and reagents

A pCAGGS/LacZ, which expresses β-galactosidase at eukaryotic cells, was inserted under the CAG promoter of a plasmid (pCAGGS). Plasmids were amplified in *Escherichia coli* DH5α and purified by a Qiagen Mega plasmid purification kit (Qiagen). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining solution was purchased from Promega. The staining solution for β-galactosidase expression cells was 20 mg/mL X-Gal (stored at −20°C), 50 m*M* potassium ferricyanide, 50 m*M* potassium ferrocyanide, and 1*M* $MgCl_2$, in phosphate-buffered saline (PBS). This solution, without X-Gal, can be prepared in advance and stored at room temperature in the dark. X-Gal was added from a stock solution just before use.

### Transfection by DDMC/DNA

Cell line 293 cells ($15 \times 10^4$ cells) were seeded on 35-mm culture dishes and incubated at 37°C in a humidified atmosphere of 5% $CO_2$. In a sterile tube, 10 μg of DNA was diluted in 270 μL of 1 × PBS (for ×1 dilute). To the DNA solution was added 14 μL of DDMC (autoclaved) having a concentration of 10 mg/mL. Then, it was mixed by brief vortexing and the growth medium was removed from the cells to be transfected. The cells were washed twice with 1 × PBS, and DDMC/DNA solution was added to cover the cells. The dish was slowly moved side to side several times to ensure complete cover of the cells, and they were incubated at 37°C for 30 min. The dish was slowly moved side to side several times during the incubation. Then, 1 mL of growth medium was added, and it was incubated at 37°C for 48 h. After the incubation, the transfection activity was determined using the X-Gal staining method.

## RESULTS AND DISCUSSION

The resulting DEAE–dextran–MMA copolymer is insoluble in water and acetone at 25°C. In view of the fact that DEAE–dextran hydrochloride is soluble in water and poly(MMA) (PMMA) is soluble in acetone, it is evident that DDMC is not a mixture of DEAE–dextran and PMMA.

The IR absorption spectrum of the copolymer shown in Figure 1 has some characteristic absorption bands at 1730 and 1000–1150 $cm^{-1}$, which are attributed to the carbonyl group of PMMA and the pyranose ring of DEAE–dextran, respectively. Thus, the resulting DDMC exhibits different solubility from DEAE–dextran and PMMA and shows the characteristic absorption in the IR absorption spectrum. From this fact, it is judged that the resulting DDMC is a graft-polymerized compound.[22]

### Reaction between DDMC and DNA

A solution of the resulting latex of DEAE–dextran–MMA copolymer was added dropwise to the DNA (EX salmon sperm) solution in order to obtain the complex of DDMC/DNA (Fig. 2). The obtained complex was insoluble in water, which is a good solvent

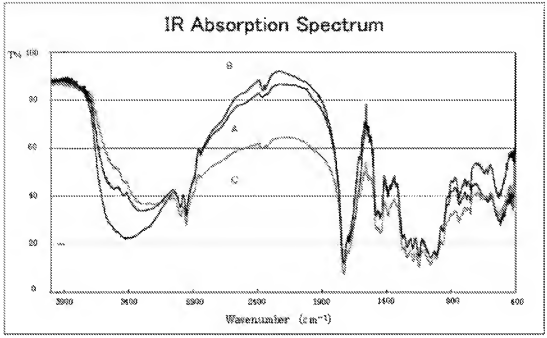


**Figure 2** IR absorption spectra of complexes between DNA and DEAE–dextran–MMA graft copolymer: DDC1/DNA complex (spectrum A), DDC2/DNA complex (spectrum B), and DDC3/DNA complex (spectrum C).

for nucleic acids. These results show that the complex between DDMC and DNA must form a polyion complex. In the case of sample DDC2, a complex between DDC2 and DNA having a 200% weight increase needed 1 h to precipitate.

The complex between DDMCs (DDC3 and DDC1) having 300 and 150% weight increases and DNA needed 0.5 and 2 h to precipitate, respectively. However, a complex between DNA and DEAE–dextran hydrochloride needed 96 h to precipitate under this condition.

Figure 1 also shows the IR absorption spectra of the resulting complex between DDC2 and DNA. The spectrum of the complex has some characteristic absorption bands at 1730, 1220, and 1000–1150 $cm^{-1}$, which are attributed to the carbonyl group of PMMA, P—O stretching vibration of DNA, and the pyranose ring of DEAE–dextran, respectively.

As shown in Table I, the complex between DDC1 having a 150% weight increase and DNA was formed in 2 h. The complex between DDMC (DDC2 and DDC3) with 200 and 300% weight increases and DNA were formed in 1 and 0.5 h, respectively. However, a complex between DNA and DEAE–dextran hydrochloride was formed in 96 h.

### Transfection by DDMC

Following the transfection protocol, transfection of HEK 293 by the DDC1, DDC2, and DDC3 samples was carried out using plasmid DNA. As shown in Figure 3, with the transfection efficiency, the transfection activity was determined using the X-Gal staining method and a value 5 times higher or more than for the starting DEAE–dextran hydrochloride was confirmed for DDMC samples DDC1 and DDC2 (but not for DDC3).

From the results, the transfection efficiency and the reaction rate of formation of the complex should increase when using DDMC hydrochloride instead of DEAE–dextran hydrochloride.

Figure 4 shows the change of the transfection efficiency when using 2 times as much as the protocol quantity of both DNA and DDMC, for example, 20 mg of DNA. As shown in Figure 4, transfection of HEK 293 by DDC1 and DDC2, carried out using 2 times as much as the protocol quantity of both DNA and DDMC shows 2 times higher efficiency than the original by the transfection activity determined using the X-Gal staining method. From the results, its cytotoxicity for the transfection should be confirmed to decrease and improve when using DDMC hydrochloride instead of DEAE–dextran hydrochloride.

DDMC transfection of cells was carried out using the following steps:

1. formation of a complex between DNA and DDMC,
2. uptake,
3. endocytosis (endosome),
4. escape from the endocytic vesicle,
5. DNA release in cytosol,
6. nuclear entry, and
7. DNA release and transcription in the nucleus.

For transfection efficiency, it is very important to examine factors such as the uptake in step 2, resistance

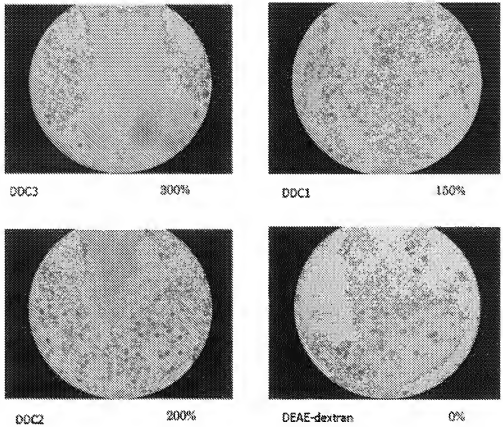


**Figure 3** The transfection of a monolayer of HEK 293 cells by the DEAE–dextran–MMA graft copolymer.

of nuclease in step 3, escape from the endocytic vesicle in step 4, nuclear targeting in step 6, and DNA release in step 7. The positively charged DEAE–dextran copolymer interacts with the negatively charged phosphate backbone of DNA. The resulting complex in step 2 is absorbed into cells by endocytosis.

The specifically designed molecular structure of DDMC having a positive charge and a hydrophilic–hydrophobic microseparated domain ensures easy entry of DNA into cells for steps 2, 3, 4, 6, and 7.

Formation of a complex between nucleic acids (DNA or RNA) and cationic graft copolymers, such as DDMC, is accomplished by a Coulomb force between the phosphoric acid of nucleic acids and the DEAE group of DEAE–dextran.

Figure 1 shows the IR absorption spectra of the resulting complex between DDMC (sample DDC2) and DNA. The spectrum of the complex has some characteristic absorption bands at 1730, 1220, 1000–1150, and 3450 $cm^{-1}$, which are attributed to the carbonyl group of PMMA, the P—O stretching vibration of DNA, the pyranose ring of DEAE–dextran, and the DEAE group of DEAE–dextran, respectively.

Figure 2 also shows the IR absorption spectra of the resulting complexes (samples DDC1, DDC2, and DDC3) between DDMC and DNA. The spectrum of the complexes has some characteristic absorption bands at around 3400 $cm^{-1}$, which is attributed to the N—H stretching vibration of the DEAE group of DEAE–dextran, following the absorption shift in the order DDC2 > DDC1 > DDC3 (to high energy). The absorption spectrum shift at around 3400 $cm^{-1}$ of the complexes may support formation of more compact structures by a Coulomb force between the phosphoric acid of DNA and the DEAE group of DEAE–dextran, to conclude DNA condensation.

This phenomenon is very interesting, because DNA is usually tightly packed in native genomes and the manner of this packaging should be expected to dominate the mechanism of gene expression.

The specifically designed molecular structure of DDMC to ensure easy entry of DNA into cells needs not only a positive charge and a hydrophilic–hydrophobic microseparated domain but also more compact structures for steps 2, 3, 4, 6, and 7. This might be the reason why the transfection efficiency of sample DDC3 with a 300% weight increase was inferior to the starting DEAE–dextran.

## CONCLUSIONS

It was recently discovered that the resulting latex of a cationic graft copolymer is superior to other high ef-

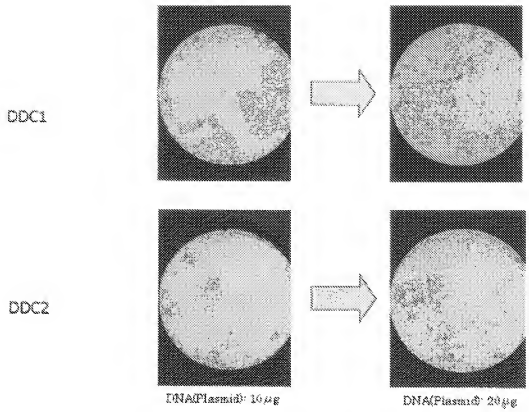


**Figure 4** The effect of the cytotoxicity of the DEAE–dextran–MMA graft copolymer on transfection of a monolayer of HEK 293 cells.

ficiency transfection reagent vectors for cells, particularly for mammalian cells.

This report is on a new class of polycationic transfection reagents based on reacting the cationic derivative of the water-soluble linear polysaccharide having hydroxyl groups with a polymerizable vinyl ester monomer in the presence of a redox initiator. The specifically designed molecular structure of the cationic graft copolymer having a hydrophilic–hydrophobic microseparated domain[24,25] ensures easy entry of DNA or RNA into cells (i.e., transfection) by condensing DNA to compact structures (graft copolymer/DNA complex or transfection complex) and endosome buffering. The high efficiency of the graft copolymer makes it a valuable tool for gene delivery or gene transfer experiments.

It is very important that these gene delivery systems consist of a first elementary step of the formation of the complex between the cationic graft copolymer thus obtained and DNA.

## References

1. Mumper, R. J.; Wang, J. M.; Claspell, J.; Rolland, A. P. Controlled Release Bioact Mater 1995, 22, 178.
2. Murata, J.; Ohya, Y.; Ouchi, T. Carbohydr Polym 1996, 29, 69.
3. Sato, T. Kobunshi 2002, 51, 837.
4. McCutchan, J. H.; Pagano, J. S. J Nat Cancer Inst 1968, 41, 351.
5. Warden, D.; Thorne, H. V. J Virol 1969, 4, 380.
6. Constantin, T.; Vendrely, C. C R Soc Biol 1969, 163, 300.
7. Mack, K. D.; Wei, R.; Elbagarri, A.; Abbey, N.; McGrath, M. S. J Immunol Methods 1998, 211, 79.
8. Onishi, Y.; Kikuchi, Y. Kobunshi Ronbunshu 2003, 60, 359.
9. Onishi, Y.; Kikuchi, Y. Kobunshi Ronbunshu 2004, 61, 143.
10. Minagawa, K.; Matsuzawa, Y.; Yoshikawa, K.; Matsumoto, M.; Doi, M. FEBS Lett 1991, 67, 295.
11. Yoshikawa, Y.; Emi, N.; Kanbe, T.; Yoshikawa, K.; Saito, H. FEBS Lett 1996, 71, 396.
12. Yamasaki, Y.; Yoshikawa, K. Kobunshi Ronbunshu 1999, 56, 772.
13. Behr, J. P.; Demeneix, B.; Loeffler, J. P.; Perez-Mutul, J. Proc Natl Acad Sci USA 1989, 86, 6982.
14. Gao, X.; Huang, L. Biochem Biophys Res Commun 1991, 179, 280.
15. Kojima, H.; Ohishi, N.; Takamori, M.; Yagi, K. Biochem Biophys Res Commun 1995, 207, 8.
16. Farber, F. E.; Melnick, J. L.; Butel, J. S. Biochim Biophys Acta 1975, 390, 296.
17. Holter, W.; Fordis, C. M.; Howard, B. H. Exp Cell Res 1989, 184, 546.
18. Haensler, J.; Szoka, F. C. Bioconjugate Chem 1993, 4, 372.
19. Kaizerman, S.; Mino, G.; Meinhold, F. Text Res J 1962, 32, 136.
20. Price, C.; Woods, D. Eur Polym Sci 1973, 9, 827.
21. Onishi, Y.; Maruno, S.; Kamiya, S.; Hokkoku, S.; Hasegawa, M. Polymer 1978, 19, 1325.
22. Onishi, Y. U.S. Pat. 4,816,540, 1987.
23. Al-Moslih, M. I.; Dubes, G. R. J Gen Virol 1973, 18, 189.
24. Onishi Y.; Maruno, S.; Hokkoku, S. Kobunshi Ronbunshu 1979, 36, 535.
25. Onishi Y. Polymer 1980, 21, 819.